Inventory of Supplementary Information

Supplementary Data

Supplementary Data

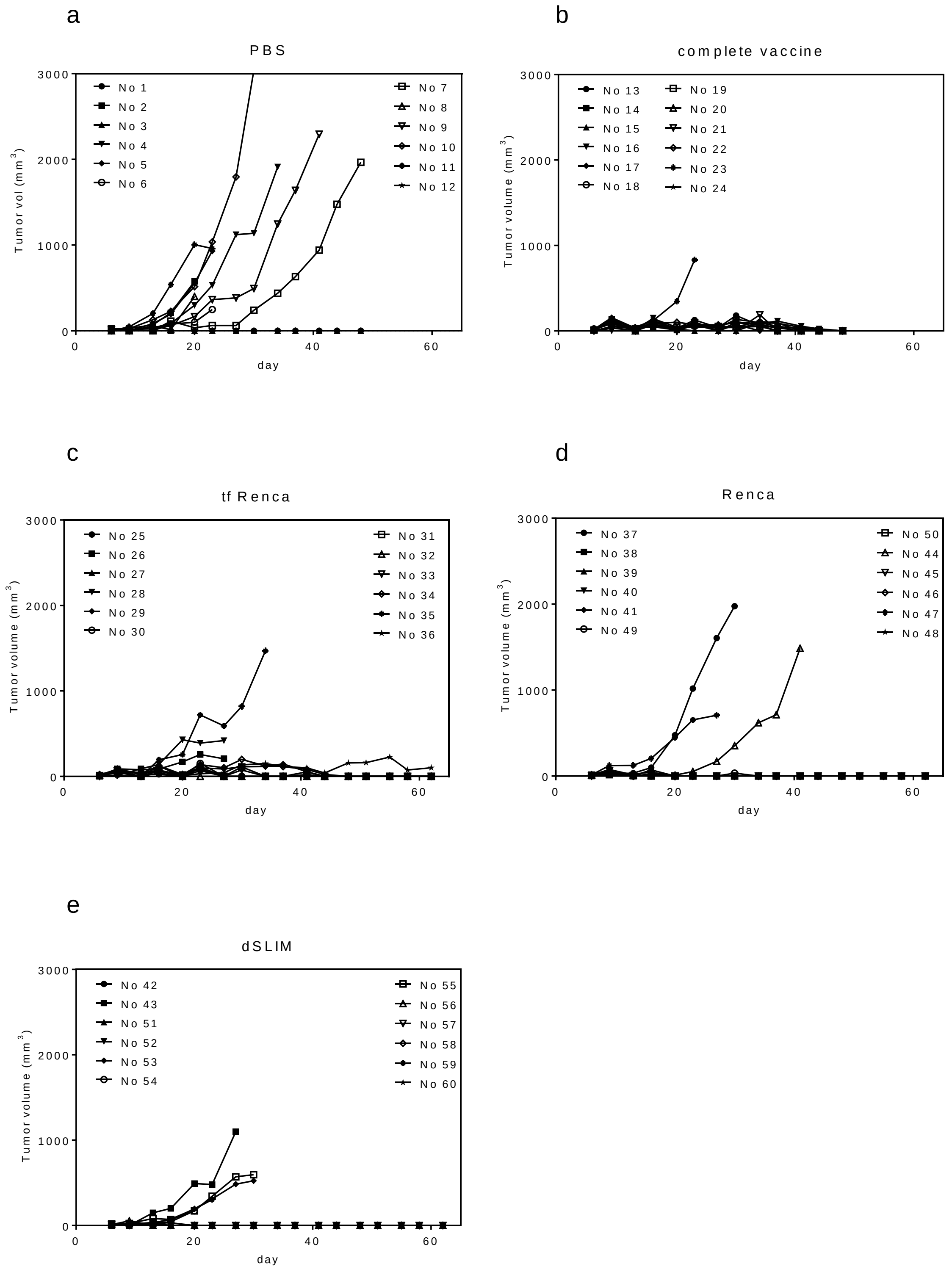


Figure S1, related to Figure 1c+d. Individual tumor growth data of mice after therapeutic vaccination (syngeneic setting) using a range of experimental variants of the murine vaccine. Mice received tumor inoculation with Renca cells and, subsequently, 4 weekly injections with vaccines (n = 12 mice/group). Individual curves of tumor growth are shown. (a) Mice treated with PBS. (b) Mice treated with the complete vaccine. (c) Mice treated with gene modified cells without dSLIM. (d) Mice treated with (irradiated) Renca cells without gene modification and without dSLIM. (e) Mice treated with dSLIM as monotherapy.

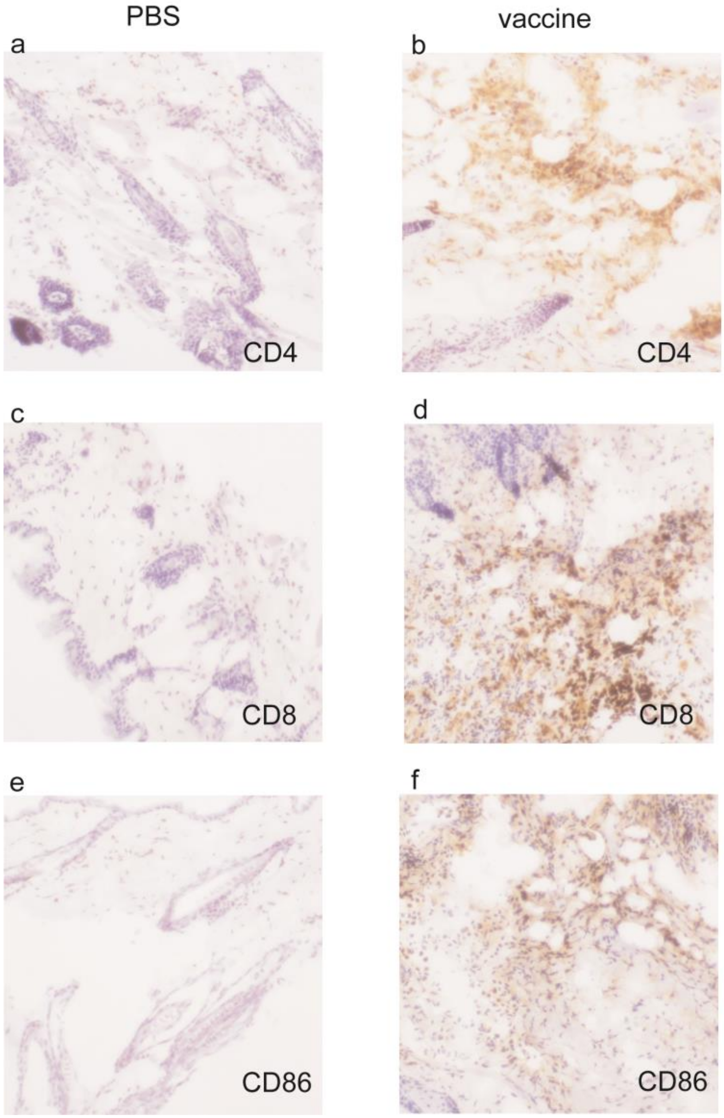


Figure S2, related to Figure 2a. Presence of CD4^{+}, CD8^{+} and CD86^{+} cells at the application sites of the murine homologue of MGN1601. Frozen tissue sections (skin) of the application sites were prepared one week after the 8th application of the murine vaccine to mice, stained for the presence of CD4^{+}, CD8^{+} and CD86^{+} positive cells and visualized by immunohistochemistry. Quantification was performed visually. Left side (a, c, e): representative pictures from PBS-treated mice scored with 0 (no staining); right side (b, d, f): representative pictures from mice treated with the murine vaccine scored with 3 (pronounced staining). Upper panel (a, b): CD4 stain, middle panel (c, d): CD8 stain, lower panel (e, f): CD86 stain.

a b

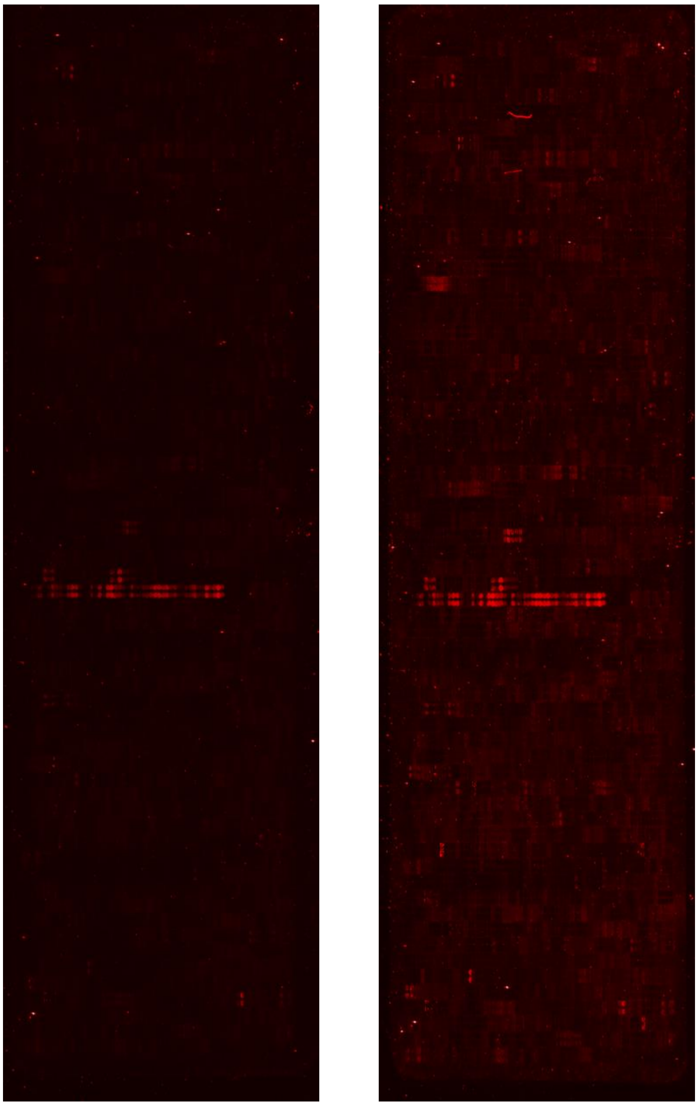


Figure S3, related to Figure 6. B**inding of serum and pre-immune serum to peptides of TAA (peptide array of patient no 9).** Peptide array consisting of 17 TAA (MET, apolipoprotein L, 1, G1/S-specific cyclin-D1, telomerase, MUC1, survivin, Myc, p53, histone H1.2, NY-ESO-1, Her-2/neu, MMP11, WT-1, G2/mitotic-specific cyclin-B1, PRAME, MAGE A3, MAGE A1) was prepared. Their sequences were transformed into 15-mer peptides with a peptide-peptide overlap of 13 amino acids resulting in 4,399 different peptides, spotted in rows from top left to down right in duplicate to the array. **(a)** Staining with pre-immune serum (1:1000). **(b)** Staining with serum (after 8 vaccinations with MGN1601) (1:1000). Intensity of binding to peptides is visualized by intensity of red staining. 1.9% of all peptides (82 out of 4399) fulfill the described requirements for "immune response": Binding intensity in serum >1000, increase in binding in serum versus pre-immune serum >2, and at least one adjacent peptide with binding intensity in serum >500.

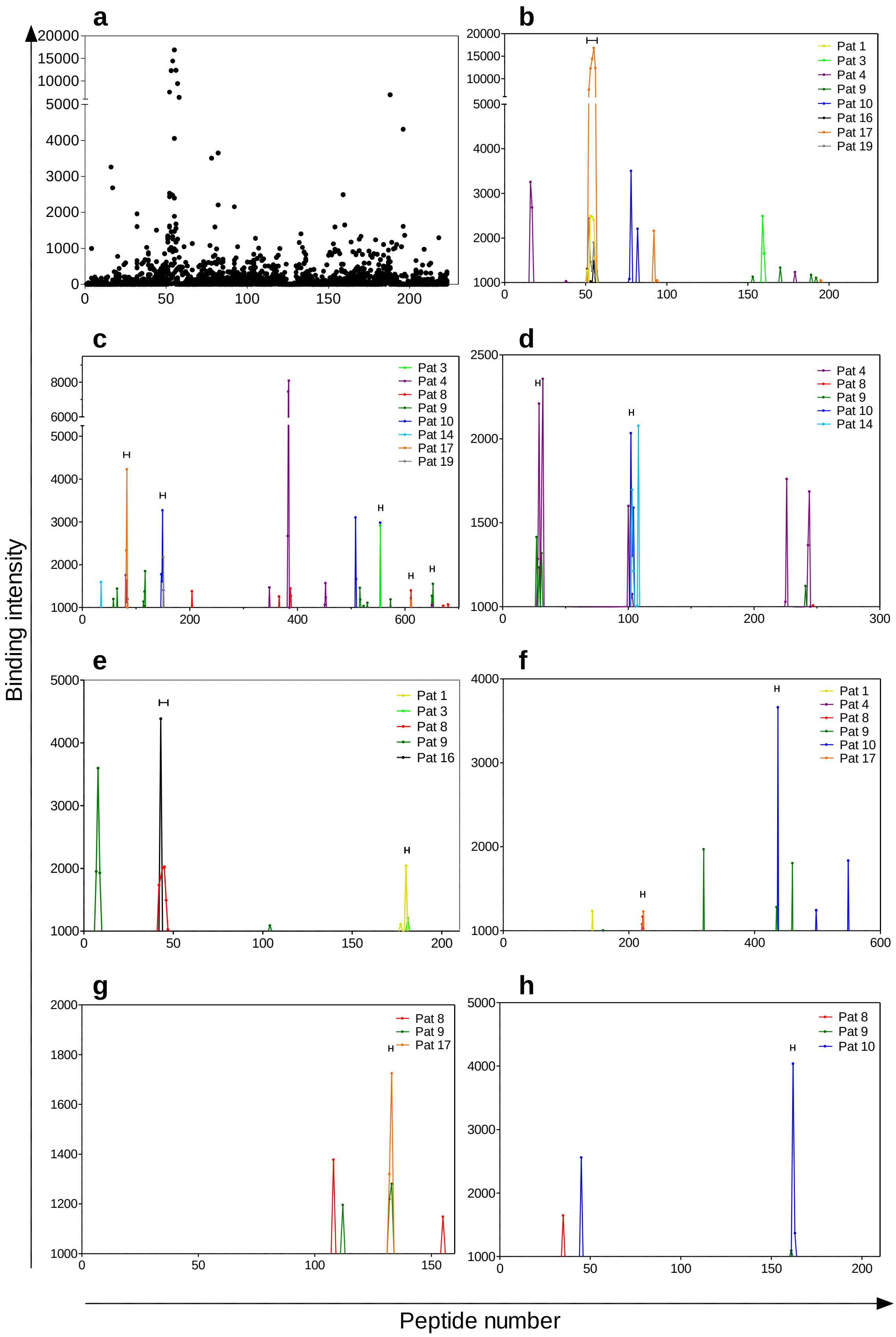
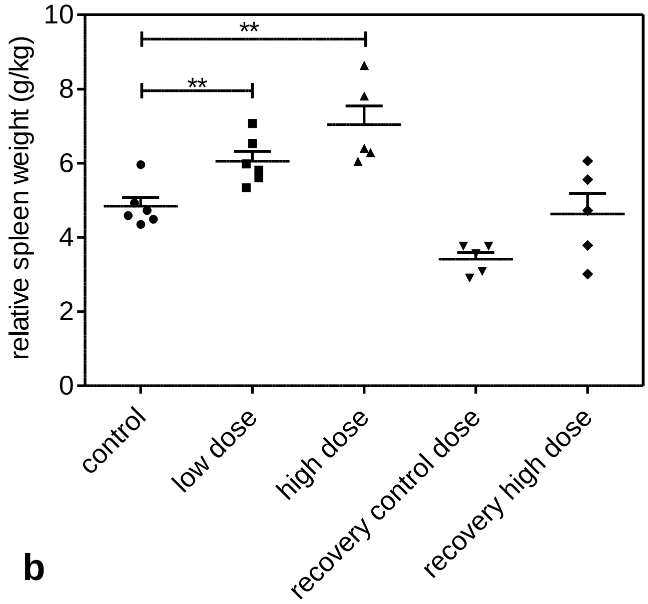


Figure S4, related to Figure 6. Induction of antibodies to TAA by vaccination with MGN1601 in mRCC patients. Sera of all PP-treated patients (N = 10) from the ASET study were collected before the first vaccination and after 8 vaccinations (week 12) and analyzed for binding intensity to 17 antigens. Each antigen was represented by successive 15-mer peptides with an overlap of 13 amino acids. Each peptide was spotted in duplicate to the array**.** (a) Binding intensities (sera of all 10 patients) against all peptides of cyclin B1 (without any criterion for immune response). (b - f) Binding intensities of sera to peptides of specified TAA are depicted when 3 immune response criteria were fulfilled: High binding intensity (> 1000) of the serum combined with a > 2-fold increase of binding signal compared to the pre-immune sera and binding intensity in serum to overlapping peptides (with at least binding signal of 500). " ⊔" indicates shared immune responses. (b) cyclin B1; (c) Met; (d) PRAME; (e) p53; (f) telomerase; (g) MAGE A1; (h) apolipoprotein L,1.

a



b

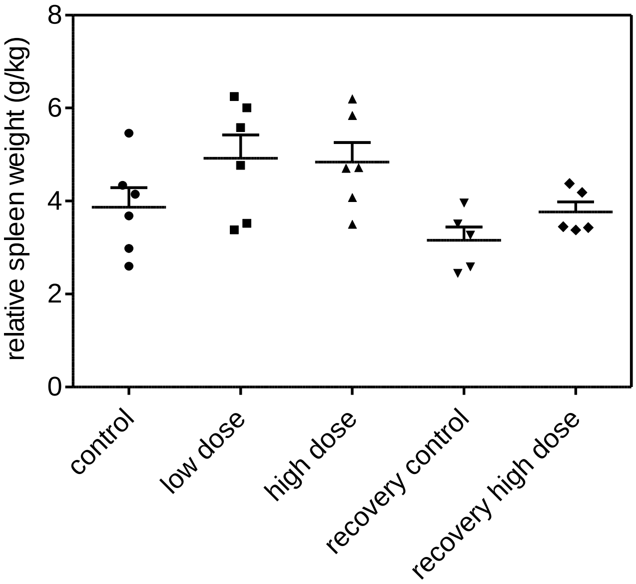


Figure S5, related to evaluation of safety. Relative spleen weight of female and male mice after repeated dosing of the murine homologue of MGN1601. Mice received 13 applications of the murine vaccine (once weekly), either low or high dose or PBS as control. One week after the last application (recovery groups: 5 weeks after the last application) spleen weight was analyzed (g spleen weight per kg body weight). (a) female mice, (b) male mice. Individual data and mean + SD are shown. ** $p < 0.01$.

Table S1, related to Figure 1c. Statistical analysis of the tumor volumes of mice after various treatment regimens[Sb].

Treatment	PBS	Complete murine vaccine	Tf Renca	Renca	dSLIM
p value versus PBS		***	***	n.s.	*
p value versus complete vaccine	***		n.s.	n.s.	n.s.

[Sb] Mice (12 per group) were inoculated with Renca cells at day 0 and vaccinated thereafter 4 times with experimental variants of the murine vaccine. Tumor volumes at the end of the study of surviving mice and at the time of death of non-surviving mice were compared (One way ANOVA, followed by Tukey's multiple comparisons test). *** $p < 0.001$, * $p < 0.05$, n.s.: not significant, $p > 0.05$.

Table S2, related to Figure 3a. mRNA expression data of TAA in B25MOL cells (log_2-based).[Sa]

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8008454	*ABCC3*	8714	10.78	10.76
8047659	*ABI2*	10152	9.92	10.04
8158725	*ABL1*	25	8.98	9.07
8082673	*ACPP*	55	5.99	5.91
7960642	*ACRBP*	84519	7.39	7.30
8174474	*ACSL4*	2182	8.27	8.56
8028524	*ACTN4*	81	12.60	12.64
8050190	*ADAM17*	6868	8.80	8.84
8095646	*AFP*	174	5.35	5.40
7921434	*AIM2*	9447	5.77	5.54
7985695	*AKAP13*	11214	8.28	8.29
8161755	*ALDH1A1*	216	12.36	12.07
7994737	*ALDOA*	226	10.87	10.97
8051241	*ALK*	238	7.28	7.25
8025402	*ANGPTL4*	51129	9.55	9.82
7927033	*ANKRD30A*	91074	5.10	5.17
7989335	*ANXA2*	302	10.76	10.82
8107330	*APC*	324	7.33	7.40
8072735	*APOL1*	8542	9.08	9.90
7961507	*ART4*	420	5.12	5.30
8048120	*ATIC*	471	10.50	10.63
8162870	*BAAT*	570	6.63	5.61
8069487	*BAGE2*	574	3.95	4.10
8081465	*BBX*	56987	9.33	9.41
8175835	*BCAP31*	10134	10.41	10.53
8023646	*BCL2*	596	8.72	8.44
8065569	*BCL2L1*	598	10.84	10.77

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8071691	*BCR*	61	8.77	8.72
8010260	*BIRC5*	332	8.77	8.95
8064031	*BIRC7*	79444	7.60	7.48
8143417	*BRAF*	673	11.06	10.87
8035304	*BST2*	684	7.92	8.02
8032380	*BTBD2*	55643	10.58	10.62
8155083	*CA9*	768	8.08	8.05
7946749	*CALCA*	796	7.66	7.46
7976200	*CALM1*	801	9.72	9.79
8035146	*CALR3*	125972	6.48	6.52
7900382	*CAP1*	10487	11.04	11.13
7951385	*CASP5*	838	5.18	5.32
8047419	*CASP8*	841	9.42	9.59
8104014	*CCDC110*	256309	6.00	5.95
8109830	*CCDC99*	54908	9.50	9.84
7968637	*CCNA1*	8900	7.29	7.26
8105828	*CCNB1*	891	9.24	9.70
7942123	*CCND1*	595	10.62	11.25
8101212	*CCNI*	10983	11.29	11.32
7953428	*CD4*	920	6.96	6.69
7939341	*CD44*	960	12.21	12.14
8016324	*CDC27*	996	9.35	9.41
7964522	*CDK4*	1019	10.43	10.48
8119088	*CDKN1A*	1026	8.44	8.73
8160441	*CDKN2A*	1029	6.81	6.63
8029086	*CEACAM5*	1048	5.92	6.08
8158976	*CEL*	1056	6.91	6.97
7996345	*CES2*	1066	9.14	8.92

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
7902702	*CLCA2*	9635	5.41	5.56
7953723	*CLEC4A*	50856	5.53	5.38
8025028	*CLPP*	8192	9.41	9.39
8153727	*CPSF1*	29894	9.08	9.09
8170580/ 8175710	*CSAG2*	728461	6.33	6.04
7903786	*CSF1*	1435	9.89	9.87
7990545	*CSPG4*	1464	6.87	6.85
8170965/ 8176149	*CTAG1A*	246100/ 1485/ 30848	8.43	8.20
8176159	*CTAG2*	30848	8.70	8.56
8079021	*CTNNB1*	1499	10.70	10.68
8174648	*CXorf61*	203413	6.06	6.16
8051583	*CYP1B1*	1545	10.76	10.29
8124196	*DCDC2*	51473	9.52	9.57
7972259	*DCT*	1638	5.22	5.24
8117900	*DDR1*	780	8.80	8.70
8040386	*DDX1*	1653	9.59	9.55
8124144	*DEK*	7913	10.20	10.48
8178377	*DHX16*	8449	8.86	8.91
7927631	*DKK1*	22943	10.60	9.73
7914194	*DNAJC8*	22826	8.57	8.83
8068422	*DOPEY2*	9980	9.27	9.02
8121588	*DSE*	29940	8.18	8.32
8083941	*ECT2*	1894	10.07	10.42
8032730	*EEF2*	1938	11.72	11.85
8016099	*EFTUD2*	9343	10.26	10.30
8132860	*EGFR*	1956	11.01	11.14
7978544	*EGLN3*	112399	9.19	9.73
8152323	*EIF3E*	3646	9.49	9.54

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8145889	*EIF4EBP1*	1978	9.11	9.33
8102817	*ELF2*	1998	8.57	8.80
7924619	*ENAH*	55740	10.46	10.37
8164269	*ENG*	2022	9.43	9.18
8041853/ 8098439	*EPCAM*	4072	9.04	9.09
7912706	*EPHA2*	1969	9.45	9.56
8081081	*EPHA3*	2042	8.20	7.47
8006906	*ERBB2*	2064	8.67	8.67
7953981	*ETV6*	2120	10.38	10.37
8143663	*EZH2*	2146	9.81	9.94
8121784	*FABP7*	2173	8.11	8.27
8096050	*FGF5*	2250	8.24	7.81
7960407	*FGF6*	2251	6.92	6.93
7970737	*FLT3*	2322	5.56	5.68
8007757	*FMNL1*	752	7.82	7.82
8058765	*FN1*	2335	12.80	12.72
7948058	*FOLH1*	2346	4.99	5.00
7971177	*FOXO1*	2308	9.10	9.00
8038192	*FUT1*	2325	7.57	7.45
8167573	*GAGE1*	???	9.73	9.65
8175234	*GPC3*	2719	6.29	6.43
8131844	*GPNMB*	10457	6.23	6.00
8171284	*GPR143*	4935	7.94	7.83
8097957	*GUCY1A3*	2982	8.94	9.01
7910124/ 8046515/ 8102860/ 8169740	*H3F3A*	3020	10.62	11.04
7909510	*HHAT*	55733	6.80	6.74
7974851	*HIF1A*	3091	6.41	6.28
8135915	*HIG2*	29923	8.91	9.66

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8124397	*HIST1H1C*	3006	9.27	9.51
8177725/ 8179034	*HLA-G*	3135	10.83	10.88
8109712	*HMMR*	3161	8.08	8.60
8072678	*HMOX1*	3162	8.42	8.46
8101449	*HPSE*	10855	6.67	6.77
8118314/ 8178086/ 8179324	*HSPA1B*	3304	9.33	9.77
7975076	*HSPA2*	3306	8.57	8.78
7931097	*HTRA1*	5654	9.59	9.63
8146092	*IDO1*	3620	5.06	5.21
8179704/ 8178435/ 8124848	*IER3*	8870	10.66	10.87
8138566	*IGF2BP3*	10643	10.13	10.04
8139488	*IGFBP3*	3486	9.21	10.09
8174598	*IL13RA2*	3598	8.42	7.93
8131666	*ITGB8*	3696	10.72	10.73
7961900	*ITPR2*	3709	8.86	8.41
8015412	*JUP*	3728	10.48	10.34
8108301	*KIF20A*	10112	8.92	9.39
8030753	*KLK3*	3818	7.19	7.02
8038655	*KLK4*	9622	7.41	7.19
7961865	*KRAS*	3845	8.31	8.32
7899753	*LCK*	3932	7.50	7.41
8025828	*LDLR*	3949	10.17	10.49
8018975	*LGALS3BP*	3959	9.97	10.11
8127380	*LGSN*	51557	4.45	4.36
7902810	*LMO4*	8543	7.03	6.99
7924107	*LPGAT1*	9926	9.03	9.11
7956301	*LRP1*	4035	8.57	8.38

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8148553	*LY6K*	54742	6.93	6.76
8175775	*MAGEA1*	4100	7.10	6.95
8175690	*MAGEA10*	4109	6.90	6.70
8175724	*MAGEA12*	4111	7.41	7.31
8175732/ 8170562	*MAGEA2*	4101	7.10	6.88
8175747	*MAGEA3*	4102	6.98	6.95
8170531	*MAGEA4*	4103	6.13	5.92
8170553	*MAGEA6*	4105	8.05	7.95
8170407/ 8175611	*MAGEA9*	4108	7.28	7.11
8166619	*MAGEB1*	4112	6.83	6.70
8166611	*MAGEB2*	4113	5.24	5.41
8175562	*MAGEC2*	51438	5.82	5.79
8172722/ 8167673	*MAGED4*	728239	7.47	7.50
7952205	*MCAM*	4162	8.58	8.39
7919751	*MCL1*	4170	10.51	10.72
7956989	*MDM2*	4193	9.22	9.39
8127854	*ME1*	4199	9.44	9.26
8135601	*MET*	4233	12.52	12.46
7991234	*MFGE8*	4240	10.73	10.39
8093171	*MFI2*	4241	8.78	8.98
8045349	*MGAT5*	4249	8.34	8.45
8154285	*MLANA*	2315	6.16	6.27
7973336	*MMP14*	4323	9.75	9.81
7995681	*MMP2*	4313	8.63	8.22
7951217	*MMP7*	4316	11.61	11.74
7998222	*MRPL28*	10573	10.65	10.60
7992071	*MSLN*	10232	7.43	7.21
7920642	*MUC1*	4582	8.35	8.35

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
7937560	*MUC2*	4583	7.36	7.27
8024255	*MUM1*	84939	8.14	8.17
8047127	*MYO1B*	4430	10.67	10.70
7943892	*NCAM1*	4684	6.66	6.36
8019857	*NDC80*	10403	7.92	8.33
7900468	*NFYC*	4802	9.61	9.86
8109975	*NPM1*	4869	6.30	6.31
7918813	*NRAS*	4893	10.84	10.86
8120967	*NT5E*	4907	8.18	8.56
8158783	*NUP214*	8012	10.05	10.19
7958895	*OAS3*	4940	8.33	8.28
7986838	*OCA2*	4948	7.13	7.17
8168316	*OGT*	8473	10.44	10.50
7956593	*OS9*	10956	10.15	9.90
7956152	*PA2G4*	5036	11.47	11.56
8167592	*PAGE4*	9506	6.04	6.09
8084963	*PAK2*	5062	9.70	9.86
8042119	*PAPOLG*	64895	8.89	9.10
8143327	*PARP12*	64761	9.87	9.63
8059301	*PAX3*	5077	6.01	5.97
8161211	*PAX5*	5079	7.63	7.52
8011759	*PFN1*	5216	12.66	12.73
8168500	*PGK1*	5230	10.53	10.62
8160297	*PLIN2*	123	10.37	10.71
8091283	*PLOD2*	5352	11.24	11.72
7984779	*PML*	5371	8.30	8.49
7989619	*PPIB*	5479	11.83	11.90
8074856	*PRAME*	23532	7.99	7.80

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
7940996	*PRDX5*	25824	10.12	10.13
8024048	*PRTN3*	5657	8.11	7.89
8161884/ 8161865	*PRUNE2*	158471	6.87	7.14
8153334	*PSCA*	8000	8.03	7.89
7962000	*PTHLH*	5744	7.28	7.46
7908553	*PTPRC*	5788	5.21	5.18
8129418	*PTPRK*	5796	9.30	9.22
8150704	*PXDNL*	137902	6.20	6.17
7981346	*RAGE*	5891	8.46	8.29
8007084	*RARA*	5914	8.69	8.75
8012598	*RCVRN*	5957	6.43	6.51
7908388	*RGS1*	5996	5.09	4.91
7908409	*RGS2*	5997	8.05	8.03
8157324	*RGS3*	5998	8.15	8.06
7906919	*RGS4*	5999	6.64	6.60
7921916	*RGS5*	8490	6.10	6.25
7918593	*RHOC*	389	11.27	11.26
8016994	*RNF43*	54894	7.87	7.93
8003679	*RPA1*	6117	9.05	9.23
8118974	*RPL10A*	4736	9.59	9.74
7998655/ 8013348/ 8172154	*RPS2*	6187	12.36	12.54
8078905/ 7918050	*RPSA*	3921	9.75	10.30
8070194	*RUNX1*	861	9.72	9.86
8170076	*SAGE1*	55511	5.21	5.28
7941478	*SART1*	9092	8.00	8.00
7966098	*SART3*	9733	8.61	8.58
7929816	*SCD*	6319	10.78	11.40
7940636	*SCGB2A2*	4250	5.99	6.09

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8138824	*SCRN1*	9805	11.00	10.90
8066513	*SDC4*	6385	10.06	9.91
8146550	*SDCBP*	6386	10.24	10.46
8049827	*SEPT2*	4735	10.70	10.57
8088065	*SFMBT1*	51460	7.41	7.35
7963970	*SILV*	6490	6.90	6.85
8036636	*SIRT2*	22933	9.07	9.20
8098904	*SLBP*	7884	9.78	9.91
8108558	*SLC35A4*	113829	10.19	10.41
7923792	*SLC45A3*	85414	7.98	7.97
8020411	*SNRPD1*	6632	7.84	7.98
8075992	*SOX10*	6663	7.56	7.45
8040070	*SOX11*	6664	8.12	8.07
8084165	*SOX2*	6657	6.84	6.77
8117165	*SOX4*	6659	8.52	8.42
7944869	*SPA17*	53340	10.01	9.93
8062190	*SPAG4*	6676	7.48	7.52
7988286	*SPG11*	80208	8.32	8.30
8167254	*SSX1*	6756	5.37	5.72
8167728/ 8172787	*SSX2*	6757	6.45	6.32
8172415/ 8167261	*SSX4*	6759	6.58	6.49
8057744	*STAT1*	6772	11.90	11.84
8015607	*STAT3*	6774	11.40	11.31
8134030	*STEAP1*	26872	7.88	8.22
8051204	*SUPT7L*	9913	9.66	9.73
7904168	*SYCP1*	8647	4.27	4.59
8180166/ 8178977/ 8125713	*TAPBP*	6892	11.13	11.11
8139100	*TARP*	6967	3.76	3.76

ProbeSetID	Gene Symbol	Gene ID	B25MOL p20	B25MOL p40
8110783	*TERT*	7015	8.33	8.29
8078350	*TGFBR2*	7048	9.24	9.30
8014974	*TOP2A*	7153	10.28	10.68
8085815	*TOP2B*	7155	7.95	8.06
7907690	*TOR3A*	64222	8.51	8.57
8012257	*TP53*	7157	9.19	9.68
8120880	*TPBG*	7162	10.13	9.83
7953508/ 7902435	*TPI1*	7167	10.21	10.46
8026513	*TPM4*	7171	9.38	9.29
8012304	*TRAPPC1*	58485	10.77	10.89
8049394	*TRPM8*	79054	5.89	5.77
8129099	*TSPYL1*	7259	10.15	10.19
8120838	*TTK*	7272	7.69	8.11
8019842	*TYMS*	7298	10.33	10.58
7948085/ 7942991	*TYR*	7299	5.49	5.11
8154367	*TYRP1*	7306	5.29	5.12
8178295/ 8124650	*UBD*	10537	7.70	7.81
8169645	*UBE2A*	7319	11.06	11.10
8119898	*VEGFA*	7422	10.51	10.93
7926368	*VIM*	7431	12.73	12.75
7947363	*WT1*	7490	8.44	8.37
8172766/ 8172757/ 8172739/ 8167710/ 8167701	*XAGE1A*	653219	6.62	6.42
8149986	*ZNF395*	55893	9.24	9.84
8125671/ 8178939	*WDR46*	9277	7.98	7.94

[Sa] mRNA was extracted from cultivated B25MOL cells (passage no 20 and passage no 40) and used in Human GeneChip® 1.0 ST (Affymetrix Inc.) analysis. Mean of two biological replicates is given.

Supplementary Materials and Methods

Cell culture. B25MOL cells were cultured in L15 –Leibovitz medium, supplemented with 10% (v/v) FBS, 1 mM glutamine, 1 mg/ml glucose, 0.1% (w/v) Na bicarbonate, 1 x MEM vitamins at 37°C in a 5% CO_2 atmosphere. Cell line B25MOL is restricted material and will not be shared. Renca (murine renal cell carcinoma cells, Cell Line Service, Eppelheim, Germany) were cultivated in EMEM medium, supplemented with 10% (v/v) FBS, at 37°C in a 5% CO_2 atmosphere.

Human antibodies: CD40-APC (BD Bioscience, Heidelberg, Germany, clone 5C3), CD44-FITC (Serotec, Releigh, NC, USA, clone Bu52), CD54-FITC (Beckman Coulter, Krefeld, Germany, clone 84H10), CD73-APC (eBioscience, San Diego, CA, USA, clone AD2), CD80-FITC (BD Bioscience, clone L307.4), CD95-FITC (BD Bioscience, clone DX2), CD105-PE (eBioscsience, clone SN6), CD146-FITC (eBioscience, clone P1H12), CD151-APC (eBioscience clone 50-6), CD154-APC (BD Bioscience, clone TRAP1), CD227 (MUC1)-AlexaFluor488 (eBioscience, clone SM3), CD326-FITC (Serotec, clone VU-1D9), CD340 (Her-2/neu)-PE (BD Bioscience, clone neu24.7), cMET-FITC (eBioscience, clone eBioclone97), EGFR-PE (BD Bioscience, clone EGFR.1). *Murine antibodies*: CD80-FITC (BD Bioscience, clone 16-10A1), CD154-PE (eBioscience, clone MR1)

Manufacturing of MGN1601.

B25Mol cells were harvested by trypsinization. Transfection mixture was prepared by combining 4 different MIDGE vectors encoding human CD80, CD154, IL-7 and GM-CSF. Transient transfection was performed by electroporation using ECM630 electroporation system (BTX), with two cuvettes in parallel (400 V, 2400 µF, 100 Ω). Each cuvette contained 800 µl cell suspension (1.8 x 10^7 cells/ml) and 36 µg DNA. Thereafter, cells were incubated in low attachment culture dishes for 30 – 90 min at 37°C for recovery. Cells were harvested, pooled, cooled to 4°C and gamma irradiated with 100 Gy (source: Cs^{137}, four cycles, 25 Gy each) to assure that further multiplication of cells is impossible. Gene-modified and irradiated cells were immediately frozen in aliquots with a predefined freezing program and stored in the vaporous phase of liquid nitrogen. For final preparation of MGN1601 two aliquots of these cells were thawed and washed three times with PBS. An aliquot of the cell suspension was counted and percentage of viable cells was determined. These cells arenamed "vaccine cells", and supplemented with dSLIM. One dose of MGN1601 comprised 1.5 ml with 3.33 mg dSLIM/ml and 6.66 x 10^6 vaccine cells/ml (in total 1 x 10^7 vaccine cells + 5 mg dSLIM), and was applied to patients within 24 h after final preparation. Out of more than 100 doses the mean number of applied cells was 0.9 x 10^7 (+/- 0.7 x 10^6) and mean percentage of viable cells was 94.5% (+/- 1.5).

For the murine homologue of MGN1601, Renca cells were used instead of B25Mol cells, and MIDGE vectors encoding the murine variants of CD80, CD154, IL-7 and GM-CSF were gene-modified by electroporation. All other steps were performed as described for MGN1601. In vitro expression data of the murine homologue of the vaccine were comparable to those of MGN1601 (Figure 4a and 4c for CD80 and CD154 expression). Cytokine secretion was analyzed as 25,295pg/4h*1 x 10^6 cells (+/-10,852) for GM-CSF and 660 ng/4h*1 x 10^6 cells (+/- 143) for IL-7 (n=7, representing about 5 x 10^9 vaccine cells).*Cell staining and flow cytometry analysis*. B25MOL cells were freshly harvested and washed, whereas vaccine cells were thawed, washed and cultivated at density of 2 x 10^6 cells per 9.5 cm^2 in tissue culture dishes. If not otherwise indicated, cultivation of vaccine cells was conducted for 4 h at 37°C. Culture supernatant was frozen and stored for analysis of cytokine concentration. For flow cytometry analysis cells were harvested either in TNE buffer (50 mM Tris, 10 mM NaCl, 0.1 mM EDTA, pH 7.4) or after trypsinization. Cells (1 x 10^6 cells/sample) were washed with PBS, thereafter with FACS-PBS (PBS supplemented with 2% (v/v) FBS, 0.1% (w/v) NaN_3 and 5 mM EDTA). Antibody or corresponding isotype control was diluted according the instructions of the manufacturer, added to harvested cells and incubated for 30min on ice in the dark. Thereafter, cells were washed two times with FACS-PBS and re-suspended in 100 µl FACS-PBS. For the Lyoplate™ Screening Panel (Human Cell Surface Marker Screening Panel, BD, Heidelberg, Germany, # 560747) analysis was performed according to the instructions of the manufacturer. For discrimination of dead cells 2 µl PI (100 µg/ml) or 10 µl 7-AAD (BD Pharmingen, final concentration: 5 µg/ml) were added. Flow cytometry acquisition and analysis were performed using a FACSCalibur (Becton-Dickinson) with CellQuest software (Becton-Dickinson). In total, 10,000 cells were acquired. Data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA). Geometric mean values were determined within the fraction of vital cells and normalized to the corresponding isotype control.

Cytokine analysis. Cell culture supernatant from cultivated vaccine cells was collected after the indicated cultivation duration (mainly 4 h) and kept frozen until analysis. Quantification of GM-CSF and IL-7 by was done by specific ELISA according to the instructions of the manufacturer (Human GM-CSF Immunoassay, #DGM00, R&D Systems, Minneapolis, MN, USA; IL-7 human ELISA kit, #ab100574, Abcam, Milton, Great-Britain). For quantification of cytokine concentration at the application sites of the murine homologue of MGN1601 in mice, biopsy homogenates were thawed and centrifuged at 10,000g at 4°C for 10 minutes. Supernatants were used at appropriate dilutions (1:10 for GM-CSF, 1:2 for IL-7) and run in duplicates. ELISA was performed according to instructions of the manufactures (Quantikine mouse IL-7 ELISA, #M7000, R&D Systems; mouse GM-CSF ELISA (#BMS612, eBioscience). Optical densities at 450 nm and 595 nm were measured with a Multimode plate reader (Mithras LB 940 Berthold Technologies).

RNA Microarray analysis. mRNA was extracted from cultivated cells either freshly harvested or after freezing (1 x 10^6 cells/sample) using RNAzol® RT according to instructions of the manufacturer. These RNA samples were used in Human GeneChip® 1.0 ST (Affymetrix Inc.) analysis. RNA processing and hybridization were performed according to the Affymetrix standard protocol. Data analysis was performed with open source statistical software R using oneChannelGUI package available within Bioconductor[1]. First, scanned images were inspected for quality control using principal component analysis and hierarchical cluster analysis. In order to obtain gene expression level the intensity values from scanned microarrays were preprocessed by using Robust Multi Array average (RMA[2]). Differentially expressed genes were identified using the bayesian adjusted t-statistics from linear models for microarray data (limma)[3]. Genes that show more than threefold change were considered as differentially expressed ($p < 0.05$). Data are MIAME-compliant deposited with accession number GSE61634.

Peptide array analysis. Each array was used in a first round for analysis of pre-immune serum and, after stripping (PBS, pH 7.4 / 0.05% Tween 20 for 2 x 1 min), in a second round for analysis of the corresponding serum and in a third round for detection of control peptides. Arrays were blocked with Rockland blocking buffer MB-070 (60 min before the first assay). Sera were diluted 1:1000 with PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland blocking buffer and incubated for 16 h at 4°C shaking at 500 rpm with a peptide array. Detection of bound antibodies was performed with a 1:5000 dilution of F(ab´)2 goat anti-human IgG(H+L) conjugated toDyLight680 (KPL) for 30 min at RT. Control spots were analyzed with monoclonal anti-HA (12CA5)-LL-Atto680 and monoclonal anti-FLAG(M2)- LL-Atto800 antibodies. Visualization was done using a LI-COR Odyssey Imaging System with scanning offset 1 mm, resolution 21 µm, and scanning intensity “green/red” of 7/7. Quantification of spot intensities and peptide annotation were done with PepSlide® Analyzer. A software algorithm calculates the standard deviation of foreground median intensities. Mean deviations of repeat determinations (N = 4399) per patient were calculated and resulted in 12.0% (+/- 2.4) for pre-immune sera and 14.0% (+/-3.9) for sera.

Treatment of NMRI mice for injection site biopsy (allogeneic setting). 48 mice were randomly assigned to one of two treatment groups. One single dose of the murine homologue of MGN1601 (1 x 10^7 (murine) vaccine cells supplemented with 5 mg dSLIM in 100 µl PBS) or PBS (control group) was distributed equally at two injection sites (left and right flank). Bubble seen after application was marked. After indicated time points (2, 4, 6, 8, 12, 26, 50 and 170 h) three mice of each group were sacrificed. Biopsies of each application site (2 biopsies per mouse) were taken**.** Tissue samples (ca. 50 µl) were homogenized in 200 µl PBS (supplemented with 1 mM EDTA, 5 µl proteinase inhibitor mix (Sigma, #P8340)), resulting in a dilution of about factor 5. Biopsy homogenates were then immediately flash frozen in liquid nitrogen. Samples were stored at -80°C until final analysis.

Treatment of NMRI mice for quantification of antibody titer and analysis of immune cells at the application site (allogeneic setting). 20 mice were randomly assigned to one of two treatment groups. 8 doses of the murine homologue of MGN1601(2 x 10^6 murine vaccine cells + 1 mg dSLIM each) or PBS (control group) were distributed equally to four injection sites (two left + two right flank) once weekly to NMRI mice. One week after the 8th application blood was collected serum was prepared and analyzed for antibodies recognizing lysate of Renca cells. For this, Renca cells were subjected to two freeze-thaw cycles followed by gentle homogenization on ice with a potter tissue homogenizer after addition of proteinase inhibitor cocktail. After three centrifugations at 10,000*g* for 10 min each the lysate was stored at -20°C until use**.** ELISA plates were coated with Renca-lysate and blocked with BSA. After 1:50 pre-dilution in PBS, sera were serially diluted 1:3 in PBS seven times (final dilution 1:109350). Immobilized antibodies were detected by biotinylated antibody against mouse IgG, followed by incubation with streptavidin-peroxidase complex. Buffer was used instead of sera to measure the blank value and titer was calculated as multiples of 18-fold blank value.

One week after the 8th application skin sections of the application sites were gained and used for immunohistochemical analysis. Frozen tissue sections were prepared and stained with HE. Presence of $CD4^+$, $CD8^+$ and $CD86^+$ positive cells were analyzed using the specific antibodies and peroxidase conjugated anti-rat antibodies. Quantification was performed visually with 4 scoring levels: 0 (no staining), 1 (slight staining), 2 (moderate staining), 3 (pronounced staining).

Treatment of NMRI mice for quantification of cytotoxicity of spleen cells and spleen weights (allogeneic setting). 46 NMRI mice were randomly assigned to one of four treatment groups (12 mice each for control group, high dose group and low dose group, 10 mice for recovery group). Mice were vaccinated either with low dose of the murine vaccine (2 x 10^6 murine vaccine cells supplemented with 1 mg dSLIM) or high dose (1 x 10^7 murine vaccine cells supplemented with 5 mg dSLIM) or PBS (control group) 13 times once weekly each. Each application was distributed equally to four injection sites (two into left and two into right flank). A recovery group that had been treated with the high doses or PBS was housed for additional 4 weeks after the 13th application.

Cytotoxicity Assay: One week (5 weeks for recovery group) after the 13th application of the murine vaccine mice were sacrificed, spleens was removed**,** separately minced and individual cell suspensions were successively passed through 200 µm and 50 µm filters. After lysis of erythrocytes spleen cells were washed twice with MEM and suspended in 1 ml MEM. 1 x 10^7 spleen cells were co-cultured for 12 hours with Renca cells in MEM in the presence of 100 U/ml IL-2 (Gibco/Invitrogen, (PMC0023) specific activity: 2.5-10 x 10^6 U/mg). Thereafter, spleen cells were washed with DMEM (10% (v/v) FBS, 25 mM EDTA 50 µM 2-mercaptoethanol) and re-suspended at 5 x 10^6 spleen cells/ml.

Target Renca cells were re-suspended in MEM and stained by addition of DiOC-18 (Sigma; #D4292-20MG, final concentration 6 µM) for 30 min at 37°C in 5% CO_2 atmosphere. The labeled Renca cells were washed with EDTA/PBS and subsequently with DMEM. Cell concentration was adjusted to 500 Renca cells/µl.

Cytotoxicity assay was performed in duplicate by co-incubation of spleen cells (5×10^5 cells), and Renca cells (5×10^4) at the effector:target ratio of 10. For control (six-fold determination) DMEM was added to 5×10^4 Renca cells (without effector cells) to measure spontaneous cell death, respective survival. After incubation for 6 hours at 37°, PI solution was added to a final concentration of 2 µg/ml and incubated for 3 min in the dark. Directly before FACS analysis calibrator beads were added to the samples. Absolute number of viable Renca cells was determined by selection of DiOC-18-positive and PI-negative gates and combined with the number of calibrator beads measured in a separate channel.

Number of viable control Renca cells (incubated without spleen cells) was used for normalization (100%). The cytotoxicity of the samples was calculated according to[4]:

$$\text{Cytotoxicity}_{\text{sample}}\ [\%] = 100 - \frac{\text{Mean of viable cells}_{\text{sample}} * 100}{\text{Mean of viable cells}_{\text{control}}}$$

Cytotoxicity of spleen cells from PBS-treated mice was low for all animals and this value was subtracted from samples in order to correct for intraday variability of the assays.

Prophylactic vaccination in mice (syngeneic setting). 20 female Balb/c mice, allocated randomly to one of two treatment groups, received either 100 µL PBS (group 1) or 100 µl of the murine homologue of MGN1601 (2×10^6 murine vaccine cells supplemented with 50 µg dSLIM) via subcutaneous injection. Application was repeated weekly for 3 more times (day 0, 7, 14, 21). On day 28 tumor inoculation with 5×10^5 native Renca cells was performed. Mice were observed for further 60 days and the tumor size was determined twice weekly by measuring the largest (D) and the smallest (d) diameter by caliper. Tumor volume was calculated by $V\ [mm^3] = D*d^2/2$.

Therapeutic vaccination in mice (syngeneic setting). 60 Balb/c mice were randomly assigned to one of 5 different treatment groups (12 mice per group). First, all mice received a tumor inoculation with Renca cells (5×10^5 cells/100 µl) at day 1. Thereafter, mice were treated weekly with 1 dose per treatment out of four experimental variants of the murine vaccine or PBS for 4 weeks (day 8, 15, 22 and 29). Tumor volume was measured twice weekly for a total of 60 days. Besides the "complete" murine vaccine (gene-modified and irradiated Renca cells plus dSLIM (2×10^6 cells plus 50 µg dSLIM per dose)) experimental variants were used. "tf Renca": gene-modified and irradiated Renca cells without dSLIM (2×10^6 cells per dose), "Renca": irradiated Renca cells without dSLIM (2×10^6 cells per dose), "dSLIM": dSLIM as single component without Renca cells (50 µg per dose) or "PBS": PBS as vehicle. Mice were classified as "tumor bearer" when their tumor size exceeds 50 mm^3 (at any time of the study) and classified as "survivors" when they were alive at the end of the study. Mice with tumor volume over 2000 mm^3 were humanly sacrificed. Tumor volumes of mice dying during the study were pursued in this calculation.

Clinical application of MGN1601 (ASET study): Clinical trial ASET, "A Phase I/II Proof-of-Principle, first in men, Multi-Center, Open-Label, Single-Arm, Non-randomized Clinical Study to **A**ssess **S**afety and **E**fficacy, of a **T**umor Vaccine Consisting of Genetically Modified Allogeneic (Human) Tumor Cells for the Expression of IL-7, GM-CSF, CD80 and CD154, in Fixed Combination with a DNA-based Double Stem Loop Immunomodulator in Patients with Advanced Renal Cell Carcinoma" (EudraCT-No: 2009-

016853-16) was carried out in accordance with local independent ethics committee standards (approved by state office for Health and Social, ethics committee, 10639 Berlin, Germany), the declaration of Helsinki, and the international conference on harmonization (ICH) Guideline for Good Clinical Practice (GCP). All patients gave written, informed consent prior to participation. 19 Patients with advanced RCC with no standard therapy available were included in this study. These patients had received various other therapies before inclusion in ASET study, including therapy with protein-kinase-inhibitors, interferons, monoclonal antibodies, interleukins or other antineoplastic products. Each dose of MGN1601, consisting of 1 x 10^7 vaccine cells supplemented with 5 mg dSLIM was administered intra-dermally into two vaccination sites, either of either upper arms, or upper tights or paraumbilically. A total of 8 applications within 12 weeks were applied for complete treatment (per protocol, PP). The first three doses were applied weekly; the following 5 doses bi-weekly. Follow-up visits were 3, 6 and 12 months, thereafter yearly for up to 5 years after the end of treatment. Patients with disease control (complete response, partial response or stable disease) by week 12 were treated in an extension phase of the study and received 5 additional applications until week 120. Out of the 19 patients, 10 received at least 8 applications (in accordance to the study plan, PP-population), 9 received less than 8 applications (non-PP population).

Statistics. Diagrams were compiled using GraphPadPrism 6.0 for Windows (GraphPad Software Inc., La Jolla, USA). Unpaired Student's t test analysis was used for single parameter comparison, for multiple comparisons Dunnett test (Figure S5) or Tukey's test (Figure 1c and 2c) were used as post-test of One way ANOVA. $p < 0.05$ was considered as significant.

Supplementary References

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